Saskatchewan

# A D F

## Agriculture Development Fund

## FINAL REPORT

**19980297**

**DEVELOPMENT OF HAPLOID PRODUCTION TECHNOLOGY APPLICABLE FOR GENETIC IMPROVEMENT OF LENTILS**

**Funded by**
**The Agriculture Development Fund**

**July 2002**
**Prepared by: PBI/NRC**

SAF
Saskatchewan Agriculture & Food
Adding value to agriculture...through research and development

102-06532/

**Development of haploid production technology applicable for genetic improvement of lentils**

**Project 98000297**

**Applicants: W.A. Keller and A.M.R. Ferrie**

**Final Report: May 1, 1999 – April 30, 2002**

## Table of Contents


Page

A. Summary ..... 2
B. Technical Report ..... 2
1. Background ..... 2
2. Objectives ..... 2
3. Results and Discussion ..... 3
a. Anther Culture ..... 3
i. Genotype and medium constituents ..... 3
ii. Bud size and growth conditions ..... 7
iii. Bud pretreatment and culture conditions ..... 8
b. Microspore Culture ..... 9
i. Sterilization ..... 9
ii. Microspore isolation ..... 9
iii. Pre-treatments on buds and plants ..... 10
iv. Media constituents ..... 11
v. Culture conditions ..... 13
4. Conclusion ..... 13
5. Further Development ..... 14
C. Personnel ..... 14
D. Equipment ..... 14
E. Project Developed Materials ..... 14
F. Acknowledgments ..... 14
G. References ..... 15
Appendices ..... 17

## A. Summary

The ultimate goal of this project was to develop haploidy technology for lentil. This involved testing a number of lentil genotypes grown under several different environments, testing various media and culture conditions, as well as determining pretreatment conditions and developmental stage of the microspore which will result in embryogenesis. Anther culture has provided valuable insight into which parameters might be most effective in microspore culture.

Experiments in anther culture focused on increasing callus production and globular embryo structure on the callus, and furthering the development of globular structure into bipolar embryos. Gamborg's B5 (R2) and L6 media containing BAP and 2,4-D were found to be most effective at producing callus. Several different Embryo Induction Media (EIM) were tested and globular callus was most effectively produced on EIM-5 and EIM-7 (Blaydes and B5 based, respectively, with 1:8 ratio of cytokinins: auxins in both) when R2 was used to generate callus. Several different media and culture conditions were tested to further the development of this preliminary embryonic structure, but no embryos were generated.

Microspore culture experiments using solid L6 media and concentrated drops of microspores on filter papers have produced cell divisions to the 6 – 8 cell stage. Several different culture conditions, pretreatments, and growth regulators were tested to further the development of the pre-embryos, but no embryos were produced.

## B. Technical Report

### 1. Background:

Both anthers and isolated microspores can be cultured *in vitro* to produce doubled haploid plants. Isolated microspore culture is the preferred technique as it is less labour intensive and is often more efficient than anther culture when both culture systems produce haploids. However, for many species, anther culture is the only method that results in doubled haploid plants. Therefore, we evaluated both culture techniques.

There are a number of factors affecting microspore/anther culture response. These include genotype, donor plant conditions, developmental stage of the pollen grain, pretreatment, media composition and culture environment. These factors were evaluated in this project.

To date, there is no published microspore/anther culture protocol for *Lens culinarus* (lentil). Doubled haploid methodology will speed up the development of lentil cultivars, especially specific market classes like red lentil which is emerging as a major export crop. In traditional breeding methodology, it can take up to five years to produce a line that is homozygous for a desired trait. With the use of doubled haploids, homozygous true breeding lines can be developed in one generation.

### 2. Objectives:

The objectives of the project were to:

a. Develop a microspore culture protocol for the production of haploid embryos or calli
b. Regenerate plantlets from microspore-derived embryos or calli
c. Double the chromosome number to produce homozygous/pure breeding lines
d. Produce seed from the doubled haploid plants
e. Transfer this technology to breeding organizations

## 3. Results and Discussion:

### a. Anther Culture

For anther culture, buds were collected and sterilized. The anthers were then dissected from the buds and plated onto solid callus induction media (CIM). Twenty anthers were placed on solid media in each 100x15 mm petri dish and incubated at 24°C in the dark. Once the calli reached 4 mm in diameter, they were transferred to embryo induction media (EIM) and returned to the dark. If globular structures were observed, the calli were then transferred to embryo development media (EDM) and placed in either the light or the dark in an attempt to obtain normal bipolar embryos.

Several different factors were evaluated in an attempt to obtain embryos from anther culture: genotype, bud size, growth condition, medium constituents, pre-treatments of the buds, and culture environment.

*i. Genotype and Medium Constituents*

During the three-year project, several different genotypes were tested for their ability to produce callus in anther culture. Since the lentil germplasm is diverse, lentil breeders at the University of Saskatchewan were contacted. They suggested that the following genotypes would give a fair sampling of the lentil germplasm:

1. 804-3
2. CDC Vantage
3. 87716R
4. Crimson
5. ILL7180 (Australian early)
6. 599-23 (CDC Robin)
7. CDC Gold
8. 512-2 (CDC Milestone)

Crosses between the genotypes that produced the most callus initially were made and tested in anther culture compared to the parent plants. No improvement in callus or embryo production was observed.

9. 1638sx (CDC Robin x ILL7180)
10. 1637sx (CDC Robin x Crimson)
11. 1657sx (Crimson x ILL7180)

The media we evaluated over the course of the project had previously been used in legume tissue culture. These are as follows:

1. Pigeon Pea (Kaur and Bhalla, 1998)
2. M1 (Soybean Callus Induction Media) (Zhao et al., 1998)
3. CCIM-1 (Chickpea Callus Induction Media) (Croser et al., 1997)
4. CCIM-2 (Chickpea Callus Induction Media) (Croser et al., 1997)
5. Finer and Nagasawa (FN) Media (Finer and Nagasawa, 1988)
6. L2 Media (Phillips and Collins, 1979)
7. Blaydes Media (Blaydes, 1966)
8. Modified Blaydes Media with excess KI (Blaydes, 1966)
9. Garden Pea (Anderson, 1999)
10. Legume Morphology Media (PBI legume group, 1999)
11. MS media with B5 vitamins (Gamborg et al., 1968)
12. Saxena Media (Saxena and King, 1987)
13. Ramana Media (Ramana et al., 1996)
14. L6 Media (Kumar et al., 1988)

For a comprehensive list of the media and their components, see Appendix 1.

The first objective was to obtain callus from the anthers. Experiments were initially conducted on six genotypes, Crimson, CDC Robin, ILL7180, CDC Vantage, 804-3, and 87716R using four different media, Pigeon Pea, M1, CCIM-1, and CCIM-2. Callus induction rates were calculated using the following equation:

$$\text{Callus Induction Rate (CIR)} = \frac{\text{\# anthers that produced callus}}{\text{Total anthers plated}} * 100$$

The results shown in Table 1 are the average callus induction rates over several experiments for all media tested.

Table 1: Callus Induction Rates for Six Lentil Genotypes

| Media | Genotype | | | | | |
|---|---|---|---|---|---|---|
| | 804-3 | CDC Vantage | 87716R | ILL7180 | Crimson | CDC Robin |
| Pigeon Pea | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.0 |
| M1 | 0.4 | 0.0 | 0.2 | 2.2 | 9.0 | 1.7 |
| CIM-1 | 0.8 | 0.0 | 0.6 | 3.2 | 5.6 | 3.8 |
| CCIM-2 | 0.8 | 0.6 | 0.2 | 3.5 | 6.5 | 0.8 |

The most responsive genotypes were ILL7180, Crimson, and CDC Robin. Callus initiated on M1 media tended to turn black and die before reaching 2 mm in diameter. Further experiments therefore focused on CIM-1 and CIM-2 media using the three most responsive genotypes.

The second objective was to increase callus production and quality, with the possibility of producing embryos. Several basal media were tested using the growth regulators present in CCIM-1 and CCIM-2 (Appendix 1). Table 2 shows the results for three basal media compared to CCIM-1 and CCIM-2 for their abilities to produce callus. The –1 and –2 refer to the growth regulators found in CCIM-1 and CCIM-2 respectively.

Table 2: Callus Induction Rates for Different Basal Media

| Media | Genotype | | |
|---|---|---|---|
| | ILL7180 | Crimson | CDC Robin |
| CCIM-1 | 3.2 | 5.6 | 3.8 |
| CCIM-2 | 3.5 | 6.5 | 0.8 |
| FN-1 | 0.4 | 1.5 | 0.4 |
| FN-2 | 3.1 | 11.5 | 0.4 |
| Blaydes-1 | 1.3 | 10.4 | 6.3 |
| Blaydes-2 | 0.6 | 17.9 | 2.1 |
| L2-1 | 1.0 | 0.0 | 0.4 |
| L2-2 | 1.7 | 6.9 | 0.4 |
| Garden Pea-1 | 0.6 | 1.3 | Not done |
| Garden Pea-2 | 0.0 | 3.1 | Not done |

As shown in Table 2, the Blaydes media improved the callus induction rate for Crimson and CDC Robin, so modifications were made to further improve callus production. The cytokinin:auxin ratio was varied such that media was obtained that had 1:10, 2:1, and 10:1 ratios, but none of these media was better than Blaydes-2 (B2). Cysteine and methionine were added to B2 media with no

improvement in the callus induction rate. Since silver nitrate ($AgNO_3$) is known for enhancing embryogenesis in some species and 1-amino-cis-cyclopentane-1,3-dicarboxcycling acid (ACC) is known to inhibit $AgNO_3$ (Biddington et al., 1988; Lentini et al., 1995; Ockendon and McClenaghan, 1993), a titration of ACC and $AgNO_3$ was conducted in B2 media. The only effect that this had was to eliminate callus production. The M1 growth regulators (Appendix 1) were added in combination with 6, 9 or 12% sucrose. Increasing the sucrose concentration reduced the CIR and did not improve callus quality or embryo production. In a related experiment, a thiadiazuron (TDZ) titration was conducted. Table 3 compares the results of 0.01, 0.1, 1.0 mg/L TDZ in Blaydes media.

Table 3: Callus Induction Rates for TDZ Titration in Blaydes Media Containing M1 Growth Regulators

| | Genotype | | |
|---|---|---|---|
| [TDZ] mg/L | ILL7180 | Crimson | CDC Robin |
| 0.01 | 5.4 | 38.3 | 19.2 |
| 0.1 | 5.0 | 38.8 | 29.2 |
| 1.0 | 2.8 | 39.3 | 31.8 |
| Blaydes-1 | 1.3 | 10.4 | 6.3 |
| Blaydes-2 | 0.6 | 17.9 | 2.1 |

Adding the M1 growth regulators significantly improved the amount of callus derived from the anthers for all genotypes (Table 3). Increasing the TDZ concentration to 1.0 mg/L (Blaydes-14) improved callus induction rate further, but callus tended to die before showing any embryoid structure. Adding 125 or 500 mg/L proline to Blaydes-14 (B14) media improved callus growth and structure, but no embryos were produced.

The carbon source was also modified, substituting stachyose or raffinose for sucrose. These carbohydrates were chosen because lentils are known to contain high concentrations of them. It was thought that because lentil plants required them, they would prove to be useful for microspore culture. While callus was produced and eventually transferred to EIM, the callus induction rate was not improved, nor were any embryos produced.

Two other basal media (Ramana et al., 1996), Ramana-1 and Ramana-2 or R1 and R2, respectively, were evaluated in anther culture and compared to B14 for its ability to produce good quality callus. Callus induction rates were improved for Crimson and ILL7180 (Table 4), and callus quality was significantly better. Smooth, yellow callus was produced which did not die before reaching the 4 mm transfer size.

Table 4: Callus Induction Rates for the Ramana Media

| | Genotype | | |
|---|---|---|---|
| Media | ILL7180 | CDC Robin | Crimson |
| Ramana-1 | 8.0 | 7.8 | 43.1 |
| Ramana-2 | 10.8 | 27.0 | 44.0 |
| Blaydes-14 | 2.8 | 39.3 | 31.8 |

Increasing the sucrose concentration in the media has been known to reduce non-microspore derived callus (i.e. anther-wall callus) therefore, experiments were conducted using four different concentrations of sucrose (3%, 6%, 9%, 12%) in the R2 media. Callus induction and growth was inhibited by the higher sucrose concentrations. Results showed that anthers on 6% sucrose have a slightly better callus quality (i.e. callus came from within the anther). After approximately six weeks, cultures on 6% sucrose showed an increased number of cracked anthers compared to

those cultures on 3 % sucrose (control). The 9% and 12% sucrose media produced very slow growing calli that often turned black before reaching 4 mm. However, if left for several months, all sucrose concentrations eventually produced callus (Table 5) and some of the slower growing calli on 9% and 12% sucrose were transferred to EIM and EDM. Increasing the sucrose was not beneficial for ILL7180, but 6% sucrose increased callus production rate for Crimson and CDC Robin (Table 5). At sucrose concentrations higher than 6%, the callus production decreased.

Table 5: Callus Induction Rates for High Sucrose R2 Media

| | Genotype | | |
|---|---|---|---|
| % Sucrose | ILL7180 | CDC Robin | Crimson |
| 3 | 10.8 | 27.0 | 44.0 |
| 6 | 3.1 | 30.6 | 64.4 |
| 9 | 1.3 | 17.5 | 40.0 |
| 12 | 0 | 13.1 | 20.0 |

Modifications were made to R2 media in an attempt to obtain embryos. Charcoal was added to R2 media with the objective that any inhibiting substances released from the anthers during anther culture would be absorbed by the charcoal, allowing embryogenesis to occur. Calli were either left on the original media or transferred to EIM. Callus left on the charcoal media did not produce embryos and both callus induction and growth were reduced. Globular structure was not formed on the charcoal media. Callus transferred to EIM from the charcoal media did not show any advanced embryo structure and no advantage of the charcoal was observed when compared to the no charcoal control.

Experiments conducted in microspore culture of lentil had shown the addition of picloram to the media to be effective in producing dividing cells. Picloram was therefore added to the B5 media (R3) to see if it could induce embryogenesis in anther culture. No advantage was observed due to the addition of picloram and callus induction was reduced.

R2 (3% sucrose) media was subsequently used to produce callus for culture condition, EIM, and EDM studies. Another basal medium, L6 containing R2 growth regulators (L6R), was tested for its callus induction rate. Induction rates were slightly improved compared to the R2 medium (Table 6), and callus quality was also improved. Calli were fast growing and often globular before transferring to EIM, thus eliminating the need for the EIM in some cases. This medium was used to generate callus for various studies, but since it was near the end of the project, the most work was done using R2 as the callus induction medium.

Table 6: Callus induction rates for L6 media

| | Genotype | | |
|---|---|---|---|
| Media | ILL7180 | CDC Robin | Crimson |
| R2 | 10.8 | 27.0 | 44.0 |
| L6R | 7.6 | 44.0 | 57.4 |

As an alternative to agar, 0.3% phytagel was added to L6R media and used to obtain callus from anther culture. While the callus induction rate was comparable to the L6R media, calli tended to be more fragile and watery. Calli were transferred to EIM as usual. Results are still pending for globular callus production on EIM.

As mentioned previously, when calli reached 4 mm in diameter, they were transferred to an EIM. Over the course of this project, several compositions were tested and evaluated for their ability to produce globular callus or other pre-embryonic structures. Appendix 2 shows the composition of all EIM tested over the three-year project. Initially, six different EIM's were tested for their ability to produce globular callus. Calli from a variety of CIM (R1, R2, B13, B11, B14 with sucrose,

stachyose, raffinose) were plated onto the six EIM's. No embryos were obtained, and if the calli were left too long on any of the media, globular structure was lost and the calli produced many roots. EIM-5 and B3 produced the highest percent of globular callus (Table 7).

Table 7: Globular callus obtained on EIM as a percent of total calli on EIM

| | Embryo Induction Media (EIM) | | | | | |
|---|---|---|---|---|---|---|
| | EIM-2 | EIM-3 | EIM-4 | EIM-5 | EIM-6 | B3 |
| Number of Globular Calli Obtained | 0 | 19 | 12 | 14 | 2 | 33 |
| Total Number of Calli Plated to Media | 109 | 132 | 112 | 65 | 73 | 154 |
| Percent Globular Callus | 0.0% | 14.4% | 10.7% | 21.5% | 2.7% | 21.4% |

The composition of B3 was similar to EIM-5 except that it contained Indole Acetic Acid (IAA). Since IAA appeared to have little effect in the other EIM tested, it was decided that the embryogenic effect was due to the other growth regulators (Appendix 2). Further experiments focused on EIM-5 to produce globular callus for embryo development studies. Most of the globular callus produced on any of the EIM's came from the Ramana based media. The growth regulators from EIM-5 were therefore added to B5 basal medium to produce EIM-7, and towards the end of the project, they were also added to L6 media (L6E). Good globular callus was obtained from both of these media, but no embryos were produced.

Zheng et al. (2001) used inducer chemicals such as 2-hydroxynicotinic acid (2HNA) to produce embryos from microspore culture of wheat. 2HNA (0.18mM) was added to L6E media and used as another EIM. While some globular calli were obtained on this media, they turned black and died before any embryos could develop.

The globular calli obtained were used for embryo development media (EDM) studies in an attempt to determine conditions that would advance the pre-embryo globular structure to bipolar embryos and eventually plants. Appendix 3 shows a summary of all media compositions tested in these studies. To date, none of them has been successful in producing embryos. Salicylic acid has been shown to enhance embryogenesis in some species (Quiroz-Figueroa *et al.* 2001; Luo et al, 2001), therefore 1 pM, 362 μM, 724 μM, and 1.1 mM salicylic acid was added to EDM-5 through EDM-12. As well, the growth regulators from EDM's 5 – 12 were added to L6 basal medium, but no embryos were produced. Most recently, a growth regulator screening study was conducted in which nine different growth regulators at seven different concentrations (EDM 13 – 21) were added to L6 medium, and evaluated for their ability to produce embryos from globular callus. No embryos were produced.

*ii. Bud size and growth condition*

Four different growth conditions were tested over the course of the project:

1. 10/5°C, 18 hour photoperiod
2. 17/13°C, 16 hour photoperiod
3. Greenhouse
4. Field

No significant difference was observed between the growth conditions. The greenhouse and field conditions did not appear to produce healthy buds, probably due to environmental stresses and insects, so these conditions were eliminated. While no difference was observed in callus induction rates between the 10/5°C and 17/13°C conditions, anthers extracted from plants in both growth conditions were cultured throughout the project since the effect of growth condition on embryogenesis was not known.

Two different bud sizes were routinely collected over the entire three-year period. As determined by DAPI staining, small buds (1 – 2 mm) tended to contain tetrads, uninucleate, and a few

binucleate microspores. Since no embryos were produced, no conclusion can be reached as to the effect of bud size on embryogenesis. Callus induction rate for the small buds was higher than when the large buds (3 – 4 mm) were used (Table 8). Since the effect on embryogenesis was not known, anthers from the large buds were cultured throughout the term of the project.

Table 8: Effect of Bud Size on CIR for Three Genotypes (as a percent of total anthers plated)

| Genotype | Media | Small Bud (1-2 mm) | Large Bud (3-4 mm) |
|---|---|---|---|
| ILL7180 | Ramana | 23.5 | 1.5 |
| | Blaydes | 5.6 | 1.8 |
| CDC Robin | Ramana | 24.4 | 10.0 |
| | Blaydes | 38.4 | 19.0 |
| Crimson | Ramana | 54.4 | 30.3 |
| | Blaydes | 55.1 | 25.7 |
| | **Average CIR** | **33.1** | **14.6** |

*iii. Bud pre-treatment and culture conditions:*

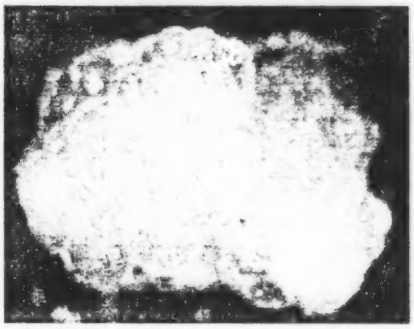
Initially buds were pre-treated at 4°C for 2 days prior to culturing the anthers. No positive effect on callus induction rate or embryogenesis was observed, so the pre-treatment was eliminated from the protocol.

A variety of experiments were conducted on the culture conditions to try to obtain embryogenesis from the anthers. None of the modifications was successful in producing embryos.

1. Anthers were cultured on small petri plates (15x60 mm) compared to the usual 15x100 mm plates. The reasoning was that if $AgNO_3$ was playing a part in embryogenesis of lentil then the small plate would increase its concentration in the culture environment.
2. Calli were left indefinitely on several of the CIM to determine if a longer period of time was necessary for embryo development. Callus grew larger and eventually produced roots. Transfer to EIM was required.
3. Temperature stress experiments were conducted on almost every CIM tested. Sealed plates were placed at 4, 15, 32, 35°C for 2 to 7 days before being transferred to 24°C. As well, some plates were kept continuously at each of these temperatures. No embryos were produced at any of the temperatures tested.
4. The size of the callus at the time of transfer to EIM was tested. Calli were transferred to EIM-7 at 1, 2, 3, 4 mm. The 1 mm calli were too small to continue development. No significant difference in globular callus production was noted between the 2 – 4 mm conditions. After this, calli were transferred between 2 – 4 mm in diameter, but no embryos were produced.
5. The effect of light on embryogenesis was tested. After transferring calli to EIM-3, sealed plates were placed in the light either immediately, after 1, 2, 3, 4, 6 weeks, or as soon as globular structures were observed. Generally, callus greened up in the light, but no globular structure was observed once calli were green. It was concluded that calli should remain in the dark until globular structure was observed.
6. Some genotypes of barley will only produce embryos if they are placed on the media in the correct orientation (Shannon et al., 1985; Hunter, 1985). Anthers from lentil were placed anterior side up, anterior side down, or partly submerged into the media on their sides. The anther orientation did not have any effect on callus induction rate, and no embryos were produced.
7. Anthers were floated on liquid L6 containing 20% ficoll or liquid B2 (Kao et al., 1991). No calli were produced on the ficoll media, but a small number of calli were produced on the B2 media. Calli did not grow larger than 2 mm in diameter, and when placed on EIM, did not develop any globular structure.

8. Suspension cultures of calli obtained on CIM were created in two separate experiments. In the first case, 1 – 2 year old callus that had not shown any development was inoculated into liquid CIM-2. In the second case, fresh callus, 2 – 4 mm in diameter was placed into liquid L6 containing 4.6 μM zeatin (L6Z) (Kumar et al., 1988). Little success was seen with the old callus, but with the fresh callus in the L6Z media, green globular pre-embryos were obtained (Figure 1). When transferred from the liquid medium and placed in the light on media containing various growth regulators (EDM's 10, 13 – 21), globular structure was lost. Calli remained green, but just grew larger without any further embryo development.

Figure 1: Green globular pre-embryos from liquid suspension culture



**b. Microspore Culture:**

Microspores were isolated from the anthers and cultured under controlled conditions in an attempt to obtain embryos. As with anther culture, a number of factors known to be important in embryogenesis were studied and modified: growth conditions, bud pretreatments, sterilization of the buds, microspore isolation, media constituents, and culture conditions.

*i. Sterilization protocol:*
One of the first problems encountered in microspore culture involved the sterilization of the buds. A simple 20% bleach sterilization was not sufficient to kill all microorganisms and produce clean cultures. The bleach concentration was increased to 50% and 100% with little effect. Ethanol, 70%, did not produce clean cultures. A 15 minute soak in 0.1% $HgCl_2$ was effective, but the microspores were killed as well as the microorganisms. Lulsdorf (1999) used a bleach/ethanol combination for chickpeas and peas. This was used for lentils, and found to be effective. The established protocol involved placing the buds in Lipshaw baskets (no more than 200 per basket) and putting the lipshaw baskets in a beaker with 20% Javex for 15 minutes on a shaker. The bleach was poured off and replaced with 70% ethanol for 10 minutes on the shaker. After the ethanol was discarded, the buds were subjected to three sterile distilled water washes of 5 minutes each.

*ii. Microspore isolation:*
The next problem encountered was that of isolating the microspores from the debris obtained from crushing the buds. After sterilization, the buds were placed in a 50 mL beaker and 5 mL of wash buffer was added. The buds were then crushed with a sterile pestle and the resulting slurry was passed through a 44 μM nitex filter into a 50 mL falcon tube. The tubes were then spun at 1100 rpm for 3 minutes. The supernatant was discarded, the pellet was resuspended in 5 mL wash buffer, and the tube was spun again. The wash step was repeated. The density of the microspores was determined by a hemacytometer and the cells were then cultured in a variety of experiments.

Crushing one or two buds and counting the microspores determined that there were about 1250 – 2917 (10/5°C) and 4167 – 6667 (17/13°C) microspores per bud. This is significantly less than the 90,000 - 120,000 microspores present in *Brassica napus* buds (Swanson, 1990). Because of this and the small size of the buds, the buds had to be thoroughly ground to release the microspores. As a result, the debris was similar in size to the microspores and passed through the filter along with the cells. Many different protocols were tested to try to separate the cells from the debris. Sucrose and percoll gradients did not solve this problem as the debris was also of similar density to the cells, so that every layer contained some microspores and some debris.

Various filter sizes were tested in a dual filter protocol. After filtering through a 44 μm filter, the slurry was passed through a 35, 20 or 10μm filters. Some extra debris was filtered out in the 35 μm filter, but not enough to give debris-free cultures. With the 10 and 20 μm filters, the majority of the debris was caught with the microspores, and it was very difficult to remove the cells from the filters, so most of the cells were lost.

In another experiment, the buds were crushed with enough pressure to release but not crush the anthers. The slurry was then passed through a mesh screen with the aim of filtering out large debris. The anthers were isolated to crush later without the presence of other plant material. This was not successful as most of the anthers got stuck in the debris and those that did pass through were difficult to crush by themselves. Again, most of the microspores were lost.

The wash buffer composition was altered, as a change in osmolality might enhance the separation of the microspores from the debris. The sucrose concentration was increased from 3% to 13% with little effect. A 0.3 M mannitol pH 4.0 wash was also tested with no improvement in the amount of debris present in the final cultures.

Decreasing the volume of wash buffer added at the crush step, beating the buds with glass beads, or blending the buds in a Waring minicup blender, increasing, decreasing, or removing spin time and rpm, and floating the anthers for natural dehiscing all proved to be ineffective at producing debris-free cultures. Eventually it was decided that after each of the spin steps, the pellet would be gently washed in wash buffer by gently swirling the tube to remove as much of the green debris as possible. In this way, a balance between microspore and debris loss was obtained. Throughout the course of the project, all cultures contained a small amount of debris.

*iii. Pretreatments on buds or plants:*

Three bud pretreatments were tested:

1. A 4-day dark treatment at room temperature had no effect on microspore viability or embryogenesis.
2. Soaking the buds in 0.3 M mannitol pH 4.0 solution at 4°C for 4 days also had no effect on microspore viability or embryogenesis.
3. A 24hr heat shock at 32, 35, or 40°C proved ineffective at inducing embryogenesis. For this pretreatment, it was discovered that the buds had to be sterilized prior to the treatment otherwise clean cultures could not be obtained.

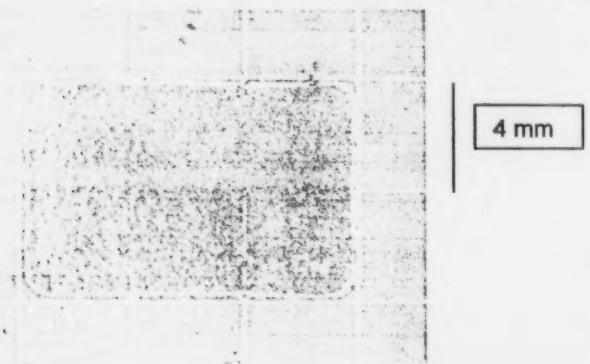
A number of plant treatments were also tested:

1. The same three growth conditions were tested as with anther culture. The poor quality of the plants and the microspores caused the elimination of the greenhouse condition. For the majority of the project, 10/5°C and 17/13°C conditions were evaluated. In the liquid culture protocol, no effect of growth condition on cell swelling or division was observed. Later in the project, a solid media protocol was tested with more success and the 17/13°C condition regularly showed more cell swelling and dividing cell masses than the 10/5°C condition.
2. A heat shock on the plants at 32 or 35°C for 6, 12, or 24 hours was tested with no effect on embryogenesis.

*iv. Media constituents:*

After the microspores were isolated, enough culture media was added to adjust the microspore density to $1.5x10^4$ cells/mL. A total of 10 mL were then plated into petri dishes. Alternatively, a few drops of culture media were added to the microspores and this was dropped onto filter papers that had been placed on solid media (Figure 2). The cell density of the microspores in this case was $3.0x10^4$ cells/grid. Plates were then sealed and placed at 24°C.

Figure 2: Microspore cake on black gridded filter paper




Media used in microspore culture mirrored those used in anther culture. Any media that was successful in anther culture was tested in microspore culture with various modifications. Unless otherwise stated, all of the modifications listed below did not produced any embryos or cell divisions:

1. A number of basal media were initially tested: N6, SH, ER, LS, WPM, NLN, B5 (Gamborg and Phillips, 1995). None of the media was better than any of the others.
2. Microspores suspended in CIM-1 were aliquoted into a filter insert and placed in a nurse culture of embryogenic *B. napus* (Topas) microspores. The filter allowed any nutrients produced by the *B. napus* embryos to pass freely into the lentil microspore culture.
3. Conditioned media has been used in pollen germination experiments (Shivanna and Rangaswamy, 1992) and for anther culture of barley (Xu et al. 1981). Lentil anthers were floated in Gamborg's B5, LS, or B2 media for 7 days. The anthers were then removed by filtration through a 44 μm filter and used for the final resuspension of the microspore pellet.
4. Lentil seeds were boiled and then crushed. The resulting slurry was then sterilized and added to B1 media for use in anther culture. Nutrients available and necessary for the growth of zygotic embryos may be required for microspore embryogenesis.
5. Activated charcoal was added to B2 and Woody Plant culture media and to the wash buffer during the crush step to absorb any toxins that may have been released into the culture from the isolation process. The addition of activated charcoal to the culture medium has been shown to enhance embryogenesis in pigeonpea microspore culture (Kaur and Bhalla, 1998).
6. *B. napus* microspore culture requires an elevated sucrose concentration for embryogenesis, so the sucrose was increased in the B2 media to 6, 9, 10, 12, 15, or 20%.
7. Amino acids are often added to microspore culture media. Asparagine and glutamine have been effective for microspore culture of peas (Lulsdorf, 2002). These amino acids as well as proline and serine were added to L6 and Blaydes media, respectively.

8. As mentioned earlier, proline had an effect on callus viability in anther culture. Proline was, therefore, evaluated in microspore culture in conjunction with increased sucrose (6 or 20%) in Blaydes media.
9. Biotin is a B vitamin known to be essential for carbohydrate metabolism in seeds. It was therefore evaluated in microspore culture of lentil by adding 25 mg/L or 33 mg/L to Blaydes basal medium.
10. Since the Ramana media were successful in anther culture for callus production, Ramana-1 and Ramana –2 medium were tested in microspore culture as well.
11. Picloram, benzyladenopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D) were added to Ramana (R3) and Blaydes basal media. While R3 media initially produced some dividing cells, this could not be repeated. Media changes to Gamborg's B5 basal, Ramana with 10, 20, 40 μM abscisic acid (ABA) or 6% sucrose Ramana media did not further the development of the dividing cells.
12. The carbon source was altered in R3 media as different species may utilize different forms of carbon. Stachyose and raffinose, for example, are known to be used in lentil. These carbon sources were therefore substituted for sucrose along with maltose, fructose, cellobiose, xylose, melazitose, and arabinose in separate experiments.
13. Sugar water was effective for microspore culture of tobacco (Vagera and Havranek, 1982), so a 3% sucrose solution and water with R3 growth regulators was tested.
14. Components of the R3 media were modified with the following:
    a. $MgSO_4$ – 250, 370, 1000 mg/L
    b. CaCl2 – 96, 300, 500 mg/L
    c. Nicotinic acid – 0, 1, 2.5, 5 mg/L
    d. thiamine•HCL – 1, 5, 10, 20 mg/L
15. pH of the R3 media was adjusted from 5.5 to 5.0, 6.0, 6.5, 7.0, 8.0, 9.0, and 10.0. In some cases, the media was changed to pH 5.5 R3 after 2, 4, or 7 days at 4, 15, 24, 30 or 32°C.
16. The strength of the Gamborg's B5 in the R3 medium was varied. Double strength, 1/10, ¾, and ½ strength were tested with no improvement in embryogenesis.
17. A sucrose starvation condition was created by substituting 6, 9, or 12% PEG (to maintain osmolality) for sucrose in the R3 medium. The stress was maintained for either, 2, 8 or 17 days at 4, 15 or 30°C. This treatment has been effective *Brassica napus* microspore culture (Ilic-Grubor et al. 1998; Ferrie et al. 2000), but was not effective in producing embryos from lentil microspores.
18. Isolated microspores were overlaid on 20% ficoll. Plates were sealed and stored at 4°C for 1 month. This stress did not induce embryogenesis.
19. Three growth regulator combinations in L6 medium were tested: basal, R3 growth regulators, 44.5 μM 2,4-D + 0.4 μM kinetin (L6-3) (Kumar et al., 1988). While no response was noted in the liquid culture with any of the growth regulator levels, L6-3 produced initial cell divisions in the filter paper protocol. When microspores were resuspended in L6-3 medium and dropped onto filter papers that were placed on L6Rp media (3% phytagel), swelling and initial divisions of the microspores was observed (Figures 3 and 4).

Figure 3: Swollen microspore

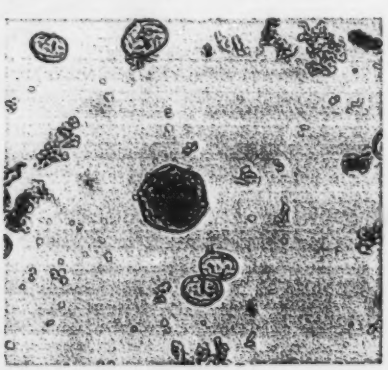
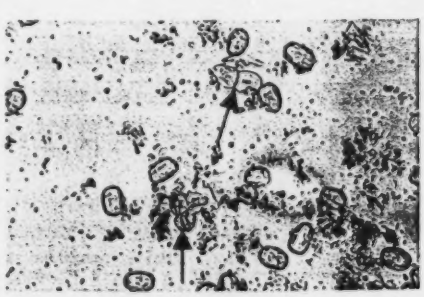


Figure 4: Initial microspore divisions



20. Microspores were washed with a cold 0.3 M mannitol solution, pH 4.0. This was effective for producing embryos in pea microspore culture (Lulsdorf, 2002). This was beneficial as dividing masses up to the 4 – 6 cells stage were observed.
21. Using the mannitol wash protocol, a growth regulator study was conducted to see if embryo development could be furthered. After one week of culture, the filter papers were removed from the L6Rp media and transferred to a fresh media containing different growth regulators. In all, 10 different growth regulators (Ancymidol, 3,6-dichloro-o-anisic acid (DICAMBA), Naphthaleneacetic acid (NAA), 2,4,5-Trichlorophenoxyacetic acid (245-T), Indole-3-butyric acid (IBA), 6-[γ,γ-dimethylallylamino]purine (IPA), Zeatin, Gibberellic acid ($GA_3$,) TDZ, Picloram) at seven concentrations used were tested but there was no effect on embryogenesis.
22. Chemicals known to enhance embryogenesis in wheat (Zheng et al. 2001) were tested in microspore culture of lentil. 2-Hydroxynicotinic acid (2HNA), benzotriazole (BT), benzotriazole-5-carboxylic acid (B5C), and violuric acid monohydrate (VAM) were tested for 2 - 8 day stresses (0.18mM) and at concentrations of 0.09, 0.18, and 0.36 mM (2-day stress). Ethylmethane sulfonate (EMS) and colchicine have been shown to enhance embryogenesis in *Brassica napus* (Ferrie, 1999; Iqbal et al. 1994). Concentrations of 0.1% and 0.2% EMS for 30 or 90 minutes were tested. As well, 0.1 mM colchicine for three days was tested. None of the chemical inducers was effective in producing embryos in lentil microspore culture.
23. A number of starvation stresses were tested. The theory was that by depriving the microspores of needed nutrients for a short time, they would be induced to undergo embryogenesis as a method of preservation. Sulphur and sucrose starvation (2 day stresses) were unsuccessful when conducted in the B2 culture medium. A nitrogen starvation was tested in B1, B2, FN, and R3 media for 2, 4, 7, 14, 21, and 30 day stresses at 32°C and 4°C. The nitrogen stress was also ineffective at producing embryos.

*v. Culture Conditions:*

The culture conditions play a large part in embryogenesis.

1. The most studied stresses in microspore culture are the incubation temperature and length of incubation. *Brassica napus* requires a 3 day stress at 32°C (Ferrie and Keller 1995). At various times throughout the project (i.e. various media) incubation temperatures of 4, 15, 18, 24, 32, and 35°C were tested for 1, 3, 4, 5, 8, 12 days. A high temperature of 60°C was used for 17 and 24 hour stresses. Microspores were also cultured continuously at each of these temperatures. Temperature seemed to have little effect on embryogenesis.
2. A freezing stress was also attempted by placing microspores at -20°C for 3 days. This proved to be too harsh a treatment, as viability testing showed no living cells after treatment.
3. It was thought that perhaps the microspore membrane was not allowing the nutrients to enter the cell, so a 1% cellulase treatment was conducted for 30 minutes, 60 minutes or overnight. No embryos were produced.
4. Microspores were placed in a vacuum overnight with no effect on cell division or swelling.
5. A short heat shock on the microspores has been shown to enhance embryogenesis in *Brassica napus* L. (Binarova et al. 1997). A 30 or 60 minute shock at 40°C was given to freshly isolated microspores. As expected, microspore viability was reduced as the heat shock time increased. No enhancement of cell division was observed compared to the control.

**4. Conclusion:**

Over three years, progress was made in both anther culture and microspore culture of lentil. Callus induction was obtained in anther culture at rates as high as 57.4% of the total anthers plated. Globular structures and early embryogenesis were obtained. In some cases, embryos

developed to the heart shaped stage. In microspore culture, cell divisions were obtained, and in the best condition, pre-embryos were observed at the 8 cell stage. To our knowledge, this is the best that has ever been reported for lentil to date.

**5. Further Development:**
Since cell divisions were observed in microspore culture, it would be beneficial to continue studying conditions that would further the development of lentil embryos. If a protocol is developed in which microspore-derived embryos and double haploid plants are produced, the benefit to lentil producers would be great. Haploidy technology can be not only be used in breeding to speed up the breeding program but can be used in mutagenesis as well as genomic research.

## C. Personnel

The ADF grant has provided part of the salary for full time work since May 3/99 for Helen Balzer (TO2 position). The allocated funding for salary was $18K per year. Marie Mykytyshyn, a Technical Officer, was hired to cover Helen Balzer's maternity leave (February 2001 – November 2001).

## D. Equipment

No major equipment was purchased for this project. Growth cabinets were rented for growing donor plants at the University of Saskatchewan and at the L.F. Kristjanson Biotechnology Complex.

## E. Project Developed Materials

One poster was presented:
"Doubled-Haploid Technology for Pulses."
Lulsdorf, M., Ferrie, A., Croser, J. Warkentin, T., and Vandenberg, B., 2002. Crop Production Show, Pulse Days, Jan 7, 8. Saskatoon.

Poster attached

## F. Acknowledgments

When groups toured the facilities, we discussed the projects currently underway, and acknowledged ADF for their support. Tours have included IRAP officers, high school students, and farmer's groups.

ADF was also acknowledged on the poster.

## G. References


Anderson, W. 1999. Washington State University, personal communication.

Blaydes, D.F. 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. Physio. Plant. 19:748 - 753.

Biddington, N. L., Sutherland, R. A. and Robinson, H. T. 1988. Silver nitrate increases embryo production in anther culture of Brussels sprouts. Annals of Botany 62:181-185.

Binarova, P., Hause G., Cenklova, V., Cordenwener, J.H.G., and van Lookeren Campagne, M.M. 1997. A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. Sex. Plant Reprod. 10:200-208.

Croser, J.S., Taylor, P.N.J., Pang, E.C.K., and Brower, J.B. 1997. Somatic embryogenesis from anthers of Australian kabuli chickpea (*Cicer arietinum*) cultivars. Proc. 3rd International Food legume Research Conference, Adelaide, Australia, September 1997.

Ferrie, A.M.R. 1999 – 2002, personal communication

Ferrie, A.M.R., Stevenson, D. and Keller, W.A. 2000. Induction of microspore embryogenesis in *B. napus* with polyethylene glycol (PEG). The XVI International Congress on Sexual Plant Reproduction. Banff. April 1 – 5, 2000. P. 45 (Abstract)

Ferrie, A.M.R. and Keller, W.A. 1995. Microspore culture for haploid plant production. Plant Cell, Tissue and Organ Culture. Fundamental methods. O. L. Gamborg and G. C. Phillips. Berlin, Springer-Verlag: 155-164.

Finer, J.J., Nagasawa, A. 1988. Development of an embryogenic suspension culture of soybean (*Glycine max* Merrill). Plant Cell Tissue Organ Cult. 15:125 - 136.

Gamborg, O.L., Miller, R.A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50:151 – 158.

Gamborg, O.L. and Phillips, G.C. 1995. Appendix A: Basal Media for Plant Cell and Tissue Culture. Plant Cell, Tissue and Organ Culture. Fundamental Methods. Berlin, Springer-Verlag: 301 – 307.

Hunter, C.P. 1985. The effect of anther orientation on the production of microspore-derived embryoids and plants of *Hordeum vulgare* cv. Sabarlis. Plant Cell Reports 4:267-268.

Ilic-Grubor, K., Attree, S.M. and Fowke, L.C. 1998. Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. Plant Cell Reports 17:329-333.

Iqbal, M.C.M., Mollers, C. and Robbelen, G. 1994. Increased embryogenesis after colchicine treatment of microspore cultures of *Brassica napus* L. J. Plant Physiol. 143:222-226.

Kaur, P. and Bhalla, J.K. 1998. Regeneration of haploid plants from microspore culture of pigeonpea (*Cajanus cajan L.*). Indian Journal of Experimental Biology, 36:736 – 738.

Kao, K.N., Saleem, M., Abrams, S., Pedras, M., Horn, D. and Mallard, C. 1991. Culture conditions for induction of green plants from barley microspores by anther culture methods. Plant Cell Reports 9:595-601.

Kumar, A.S., Gamborg, O.L. and Nabors, M.W. 1988. Plant regeneration from cell suspension cultures of *Vigna aconitifolia*. Plant Cell Reports 7:138 – 141.

Lentini, Z., Reyes, P., Martínez, C.P. and Roca, W.M. 1995. Androgenesis of highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Science 110:127-138.

Lulsdorf, M. 1999 - 2002. University of Saskatchewan, personal communication.

Luo, J., Jiang, S., and Pan, L. 2001. Enhanced somatic embryogenesis by salicylic acid of *Astragalus adsurgens* Pall.: relationship with $H_2O_2$ production and $H_2O_2$-metabolizing enzyme activities. Plant Science. 161:125 - 132.

Ockendon, D.J. and McClenaghan, R. 1993. Effect of silver nitrate and 2,4-D on anther culture of Brussels sprouts *Brassica oleracea* var. *gemmifera*. Plant Cell Tissue and Organ Culture 32:41-46.

Phillips, G.C. and Collins, G.B. 1979. In vitro tissue culture of selected legumes and plant regeneration from callus of red clover. Crop Science 19:59-64.

Quiroz-Figueroa, F., Mendez-Zeel, M., Larque-Saavedra, A., and Loyola-Vargas, V.M. 2001. Picomolar concentrations of salicylates induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture. Plant Cell Reports 20:679-684.

Ramana, R.V, Venu, C., Jayasree, T., and Sadanadam, A. 1996. Direct somatic embryogenesis and transformation in *Cicer arietinum* L. Ind.J. Exp. Bio. 34:716 – 718.

Saxena, P.K. and King, J. 1987. Morphogenesis in lentil: Plant regeneration from callus cultures of Lens culinaris Medik. via somatic embryogenesis. Plant Sciences 52:223-227.

Shannon, P.R.M., Nicholson, A.E., Dunwell, J.M., and Davis, D.R. 1985. Effect of anther orientation on microspore-callus production in barley (*Hordeum vulgare* L.). Plant Cell Tissue Organ Culture 4:271-280.

Shivanna, K.R. and Rangaswamy, N.S. 1992. Pollen Biology: a laboratory manual. Springer-Verlag, New York, Chapter 4, p24-26.

Swanson, E.B. 1990. Microspore culture in *Brassica*. In: Methods in Molecular Biology. Eds: J.W. Pollard and. J.M. Walker, Humana Press. Vol.6, Plant Cell and Tissue Culture: pp159-169.

Vagera, J. and Havranek, P. 1982. Effect of minimization of culture medium on the process on androgenesis *in vitro* (*Nicotiana tabacum* L. var. White Burley, *Datura innoxia* Mill.). Biochem. Physiol. Pflanzen 177:266-274.

Xu, Z.H., Huang, B., and Sunderland, N. 1981. Culture of barley anther in conditioned media. J. of Exp. Bot 32:767-778.

Zhao, G., Liu, Y., Yin, A. and Li, J. 1998. Germination of embryo in soybean anther culture. Chinese Science Bulletin 43:1991-1995.

Zheng, M.Y., Liu, W., Weng, Y., Polle, E. and Konzak, C.F. 2001. Culture of freshly isolated wheat (*Triticum aestivum* L.) microspores treated with inducer chemicals. Plant Cell Reports 20:685 – 690.

## Appendices

### *Appendix 1 – Callus Induction Media Constituents*

| Growth Regulators | Media | | | | | | |
|---|---|---|---|---|---|---|---|
| | CIM-1 | CIM-2 | M1 | Blaydes | Ramana | | L6 |
| Base | MS | MS | MS | Blaydes | B5 R1 R2 | L6 | 2.0 |
| 2,4-D | 2.0 | 0.1 | 2.0 | | | 2 | |
| Kinetin | 0.2 | | | 0.5 | | | |
| Indoleacetic Acid | | 1.0 | | 5.0 | | | |
| Naphthaleneacetic Acid | | 2.0 | 2.0 | | | | |
| TDZ | | | 0.01 | | | | |
| 2,4,5-T | | | | | 2 | | 1.0 |
| Benzyladenopurine | | 2.0 | 1.0 | | 1 | 1 | |

*all concentrations are in mg/L

### *Appendix 2 – Embryo Induction Media Constituents*

| Growth Regulators | Media | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | EIM-1 | EIM-2 | EIM-3 | EIM-4 | EIM-5 | EIM-6 | EIM-7 | Blaydes-3 | L6E |
| Base | MS | B5 extra $NH_4NO_3$ | Blaydes | Blaydes | Blaydes | Blaydes | B5 | Blaydes | L6 |
| $AgNO_3$ | 1,5,10 | | | | | | | | |
| Mannitol | 0.3% | | | | | | | | |
| 2,4-D | | | 0.1 | | 0.1 | | 0.1 | 0.1 | 0.1 |
| Naphthaleneacetic Acid | | | 2.0 | | 1.5 | | 1.5 | 2.0 | 1.5 |
| Indoleacetic Acid | | 0.25 | | 2.0 | | 1.6 | | 1 | |
| Benzyladenopurine | | 1.0 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |

*all concentrations are in mg/L unless otherwise stated

### *Appendix 3 – Embryo Development Media Constituents*

B5 basal medium was used except where otherwise noted:

1. 500mg/L $NH_4NO_3$; 70mg/L glutamine or glutathione (EDM-2)
2. Blaydes; 5% mannitol or sorbitol (EDM-3)
3. 2.64mg/L Abscisic acid (EDM-4)
4. 0.2 mg/L BAP (EDM-5)
5. 0.1 mg/L $GA_3$ (EDM-6)
6. 0.5 mg/L $GA_3$ (EDM-7)
7. 1.0 mg/L $GA_3$ (EDM-8)
8. 0.4 mg/L BAP (EDM-9)
9. 0.6 mg/L BAP (EDM-10)
10. 0.8 mg/L BAP (EDM-11)
11. 1.0 mg/L BAP (EDM-12)
12. Ancymidol - 0.2, 0.4, 1, 2, 4, 10, 20 mg/L (EDM-13)
13. NAA, Dicamba, IPA, IBA, Picloram, 2,4,5-T, Zeatin – 0.1, 0.2, 0.5, 1, 2, 5, 10mg/L (EDM 14 – 20)
14. TDZ – 0.05, 0.1, 0.25, 0.5, 0.5, 1, 2.5, 5 mg/L (EDM-21)

# Doubled-Haploid Technology for Pulses

Monika Lülsdorf

Bert Vandenberg

Tom Warkentin

Monika Lülsdorf[1], Alison Ferrie[2], Janine Croser[3], Tom Warkentin[1], and Bert Vandenberg[1]

[1]Crop Development Centre, University of Saskatchewan,Saskatoon, SK, S7N 5A8 Canada

[2]Plant Biotechnology Institute, NRCC, Saskatoon, SK, Canada

[3]CLIMA, University of Western Australia, Nedlands, WA, Australia

Alison Ferrie


## ABSTRACT

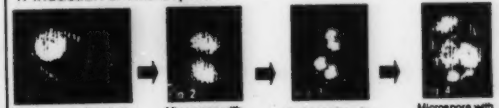

Doubled-haploid technology using anther or microspore culture is a powerful breeding tool for the improvement of crops. Microspores are immature pollen grains that contain only a haploid set of chromosomes. When they are cultured under sterile conditions, they can develop into embryos and subsequently be regenerated into haploid plants. Spontaneous or induced doubling of chromosomes will then allow regeneration into completely homozygous diploid plants. The main advantage of this technology is that one can rapidly fix traits in the homozygous condition, eliminating 3 to 4 generations of selfing. In addition, selection becomes more efficient, recessive genes can be more easily identified, and genetic manipulation is also easier at the haploid level.

This technology is now commonly used in crops such as canola, barley and wheat for the development of advanced breeding lines and cultivars (e.g. Quantum, Q2, Dupin, Mingo, or McKenzie). However, this technology is not yet available for pulses. Research is in progress at the Crop Development Centre (University of Saskatchewan) and at the Plant Biotechnology Institute (NRCC Saskatoon) to develop this doubled-haploid technology for pea, chickpea, and lentil. Microspore-derived embryos have been obtained from peas and chickpeas. However, more research is required in order to develop an efficient and reliable system for these crops.

The availability of doubled-haploid technology for pulses would have a significant economic impact. Speeding up the breeding process for pulses and improving selection would allow pulse growers faster access to improved cultivars.


## Pea

### 1. Induction of Microspores



Microspore nucleus just prior to first division.

Microspore with 2 symmetrical nuclei

Nuclei continue to divide

Microspore with multiple nuclei

Microspores are immature pollen grains that normally follow the gametophytic pathway and develop into mature pollen. However, under stress conditions such as heat or cold, they can be induced to follow a different (sporophytic) pathway that leads to the development of embryos. Under stress, microspore nuclei undergo several symmetrical divisions (see Figs. 1, 2, 3, 4 for pea, Figs. 11, 12 for chickpea) with associated cell divisions.

### 2. Embryo Development

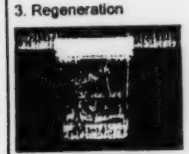
Pro-embryos with suspensor in early globular stage.

Globular to heart-shape stage embryo.

Mature pea embryo on plate

Growth regulators in the medium then induce those cells to differentiate and to develop into pro-embryo like structures (Fig. 5 for pea, Fig. 13 for chickpea). Further embryo development follows the same stages as in 'regular' embryogenesis from globular (Fig. 6 for pea, Figs. 14, 15, 16 for chickpea) to heart-shaped stage (Fig. 17 for chickpea), and to the cotyledonary stage (Fig. 7 for pea, Fig. 10 for lentil, Fig. 18 for chickpea).

### 3. Regeneration

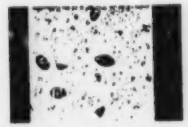


Pea plantlet regenerated from microspore

Mature embryos can then be regenerated into plantlets (Fig. 8 for pea).

## Lentil

### 1. Induction of Microspores



Isolated lentil microspore

### 2. Embryo Development

Potential embryo developed from isolated lentil microspores


## Acknowledgement

These projects were funded by AFIP (Agri-Food Innovation Fund),Western Grains Research Foundation (WGRF), CARDS (Canadian Adaptation for Rural Development in Saskatchewan), SADF (Saskatchewan Agriculture Development Fund), and CFI Canadian Foundation for Innovation).

We thank Helen Baker, Anne Allen, Tim Desmond, and Gordana Radosavljevic for their technical assistance.


## Chickpea

### 1. Induction of Microspores

Mono- and bi-nucleate microspore
Note symmetrical division i.e. 2 similar nuclei

### 2. Embryo Development

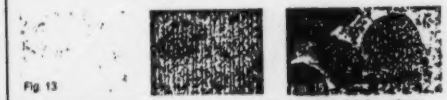


Fig. 13

Early stage pro-embryo with attached suspensor

Early globular embryo with attached suspensor

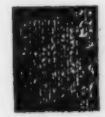
Late stage globular embryos

Fig. 18

Globular embryos on plate

Fig. 17

Heart-shaped stage embryo



Cotyledonary stage embryo

Doubled-haploid technology for pulses is still in its early stages of development. Currently, we are able to obtain a large number of globular embryos from pea and chickpea within 7 days of culture. However, the switch from the globular stage to the mature embryo is still difficult to initiate and we rarely obtain plantlets. More research is required to improve the development of a microspore culture protocol for pulses. Results obtained from our experiments so far indicate that there is a potential for obtaining a protocol that will be efficient and cost effective.